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Short Telomere Load, Telomere Length, and Subclinical Atherosclerosis

The PESA Study

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ABSTRACT

BACKGROUND Leucocyte telomere length (LTL) shortening is associated with cardiovascular ischemic events and mortality in humans, but data on its association with subclinical atherosclerosis are scarce. Whether the incidence and severity of subclinical atherosclerosis are associated with the abundance of critically short telomeres, a major trigger of cellular senescence, remains unknown.

OBJECTIVES The authors conducted a cross-sectional exploration of the association between subclinical atherosclerosis burden and both average LTL and the abundance of short telomeres (%LTL<3 kb).

METHODS Telomere length was assessed by high-throughput quantitative fluorescence in situ hybridization in circulating leukocytes from 1,459 volunteers without established cardiovascular disease (58% men, 40 to 54 years of age) from the PESA (Progression of Early Subclinical Atherosclerosis) study. Subclinical atherosclerosis was evaluated by coronary artery calcium scan and 2-dimensional/3-dimensional ultrasound in different aortic territories. Statistical significance of differences among multiple covariates was assessed with linear regression models. Independent associations of telomere parameters with plaque presence were evaluated using general linear models.

RESULTS In men and women, age was inversely associated with LTL (Pearson's $r = -0.127$, $p < 0.001$) and directly with %LTL<3 kb (Pearson's $r = 0.085$; $p = 0.001$). Short LTL reached statistical significance as a determinant of total and femoral plaque in men, but not in women. However, this association was not sustained after adjustment for age or additional adjustment for cardiovascular risk factors. No significant independent association was found between %LTL<3 kb and plaque burden. Serum-oxidized low-density lipoprotein levels were directly associated with %LTL<3 kb in men ($p = 0.008$) and women ($p < 0.001$).

CONCLUSIONS In a cross-sectional study of a middle-aged population, average LTL and short telomere load are not significant independent determinants of subclinical atherosclerosis. Longitudinal follow-up of PESA participants will assess long-term associations between telomere length and progression of subclinical atherosclerosis.
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ABBREVIATIONS AND ACRONYMS

%LTL<3 kb = percent short telomeres (<3 kb)

2D = 2-dimensional

3D = 3-dimensional

CACS = coronary artery calcium score

CT = computed tomography

CVD = cardiovascular disease

DNA = deoxyribonucleic acid

HT-QFISH = high-throughput quantitative fluorescence in situ hybridization

IMT = intima-media thickness

LTL = leucocyte telomere length

ox-LDL = oxidized low-density lipoprotein

TL = telomere length

Atherosclerosis and associated myocardial infarction or stroke are the major cause of morbidity and mortality in industrialized countries, and the economic, social, and human costs associated with treating cardiovascular disease (CVD) are painfully high (1). Our previous results revealed highly prevalent subclinical atherosclerosis in almost one-half of 4,066 participants in the PESA (Progression of Early Subclinical Atherosclerosis) study, all of whom were free from established CVD; moreover, noninvasive imaging demonstrated frequent atherosclerosis in subjects at low risk according to traditional risk scores (2). To improve diagnosis and prevention, it is thus urgent to refine classic CVD risk scores and identify new biomarkers of atherosclerotic disease initiation and early progression.

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Attrition of telomeric DNA is assumed to be a biomarker of aging and health status (3). Because age is a crucial determinant of CVD (4), the possibility that telomere attrition might predict cardiovascular risk has awakened tremendous interest. Telomeres are capping deoxyribonucleic acid (DNA)–nucleoprotein complexes that protect the ends of all eukaryotic chromosomes from degradation, unwanted recombination, or fusion (5). In most somatic cells, including leukocytes, telomeric DNA is shortened with every cell division throughout life from right after birth, in part due to inactivation of the telomere-elongating polymerase telomerase. When telomeric DNA becomes critically short, cells trigger an irreversible DNA damage response that leads to cellular senescence and, eventually, to apoptosis, thus compromising tissue repair capacity and function (6). Although human leukocyte telomere length (LTL) is predominantly determined by inheritance, it can be influenced by stresses during pregnancy and exhibits high interindividual variability at the same age (7,8). Telomeric dysfunction and attrition can be caused by genetic mutations (9) or cardiovascular risk factors (e.g., inflammation, oxidative stress, lack of estrogens, insulin resistance, smoking, obesity, alcohol abuse, sedentary life-style, psychological stress) (10–12).

Numerous epidemiological studies have investigated the relation between LTL and cardiovascular ischemic events, but the outcomes are often conflicting or too heterogeneous, as recently revealed in 2 independent meta-analyses (13,14). In their meta-analysis of 27 studies, D'Mello et al. (13) found a significant association between shortened LTL and myocardial infarction, type 2 diabetes mellitus, and stroke. In their meta-analysis of 24 studies, many overlapping those of D'Mello et al. (13), Haycock et al. (14) also showed an inverse association between LTL and nonfatal myocardial infarction, coronary heart disease death, and coronary revascularization, but the association with cerebrovascular disease was less certain. Paradoxically, D'Mello et al. (13) did not find a significant association between LTL and coronary artery disease (a secondary outcome defined as angina and nonfatal ischemic heart disease), suggesting that LTL attrition is a marker of plaque rupture and associated ischemic events.

An interesting question is whether shortened LTL is a prognostic biomarker for the early identification of subjects at high risk of developing CVD before symptoms appear. Compared with the wealth of information available on short LTL and established CVD (e.g., myocardial infarction or stroke), few epidemiological studies have focused on possible associations between telomere length (TL) and subclinical (asymptomatic) atherosclerosis. The Asklepios cohort study is the largest reported to date (2,509 volunteers free from established CVD, approximately 35 to 55 years of age) (15). The main conclusion of the Asklepios study was that shortened LTL is not a substantial determinant of preclinical atherosclerosis in either sex, and that the association between LTL and CVD cannot be explained by increased predisposition to atherosclerosis as a consequence of shorter inherited telomeres (15). Atherosclerosis in the Asklepios study was assessed in carotid and femoral arteries by intima-media thickness (IMT) and by vascular echography (absence/presence of plaque), but the potential association between LTL and plaque volume was not assessed. Moreover, to the best of our knowledge, the possible association between CVD and short telomere load has not yet been investigated, despite firm evidence showing that the accumulation of a few critically short telomeres in a cell can trigger cellular senescence (16–20). Here, we performed a cross-sectional study using high-throughput quantitative fluorescence in situ hybridization (HT-QFISH) (18) to quantify the percentage of short telomeres (%LTL <3 kb) and average LTL in a subset of 1,459 healthy volunteers in the PESA cohort, and investigated the possible association of these parameters with plaque burden measured by 3-dimensional (3D) sonography in different vascular territories.

METHODS

STUDY DESIGN AND POPULATION. The PESA study rationale and design were described elsewhere (21).

In brief, PESA-CNIC-Santander is a prospective cohort study including 4,066 asymptomatic employees at the Banco de Santander Headquarters in Madrid (Spain). Participants were 40 to 54 years of age at recruitment (between June 2010 and February 2014) and were free of established CVD. Peripheral blood samples suitable for HT-QFISH (see the "Telomere Measurements in Leukocytes" section) were available only from the last 1,500 participants recruited to the PESA study. Of these, complete data were available for 1,459, who were therefore included in the present study. This subgroup shares similar main characteristics with the overall PESA study cohort (e.g., sex, age) and exhibited slightly lower subclinical atherosclerosis prevalence (3% to 7% lower). Employees were invited to participate during their regular annual medical checkup by the company's medical services. Subjects with established CVD or any other condition reducing life expectancy were excluded from the study. The study visits include a clinical interview, physical examination (anthropometric measures, blood pressure, and heart rate), a fasting blood draw and urine sample, life-style questionnaires, accelerometry assessment of physical activity, electrocardiogram, and assessment of subclinical atherosclerosis by noninvasive vascular imaging tests, including 2-dimensional (2D)/3D vascular ultrasound and computed tomography (CT) for coronary artery calcium score (CACS) measurement. The ethics committee of Instituto de Salud Carlos III in Madrid, Spain, approved the study protocol, and written informed consent was obtained from each participant before enrollment.

LIFE-STYLE AND CVD RISK FACTORS. Traditional risk factors, such as smoking habits and a diagnosis of hypertension, diabetes, or dyslipidemia, were collected as part of each participant's medical history. Blood pressure was measured at rest with an automatic oscillometric sphygmomanometer (OMRON HEM-907, OMRON Healthcare, Kyoto, Japan). Anthropometric measures were obtained following a standardized procedure. Body mass index was calculated as body mass divided by the squared height (kg/m^2). Fasting blood and urine samples were collected after ≈8 h of fasting and tested for serum glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, C-reactive protein, whole-blood glycosylated hemoglobin (HbA_{1c}), and markers of inflammation, such as oxidized low-density lipoprotein (ox-LDL) and urinary isoprostanes. Dietary intake was assessed with a computerized questionnaire (Dietary History-Enrica), conducted by trained dieticians, designed to record habitual food intake over the previous year.

VASCULAR ULTRASOUND. All ultrasound recordings were analyzed at the PESA Core Imaging Laboratory at the CNIC. Imaging studies included vascular 2D ultrasound of carotid arteries, infrarenal aorta, and iliofemoral arteries; 3D ultrasound of carotid and femoral arteries; and CACS by CT scan. Vascular ultrasound was performed with a Philips iU22 ultrasound station (Philips Healthcare, Bothell, Washington), with adapted scanning protocols (22). A 2D high-resolution linear array transducer was used to assess the presence of plaques, and a 3D high-resolution transducer was used to assess plaque volume and, subsequently, to estimate the total atherosclerotic burden. Plaque burden was quantified with a semiautomatic 3D plaque analysis system engineered by Philips (Xcelera system and QLAB-VPQ [vascular plaque quantification]). Plaques were defined as any focal protrusion of more than 0.5 mm or more than 50% thicker than the surrounding intima-media (23). The QLAB-VPQ system estimated plaque burden by summing plaque areas and plaque volumes from successive cross-sectional scans of vascular segments containing atherosclerotic lesions. CACS was estimated by noncontrast electrocardiographic-gated prospective acquisition with a 16-slice Brilliance CT scanner (Philips Healthcare, Andover, Massachusetts).

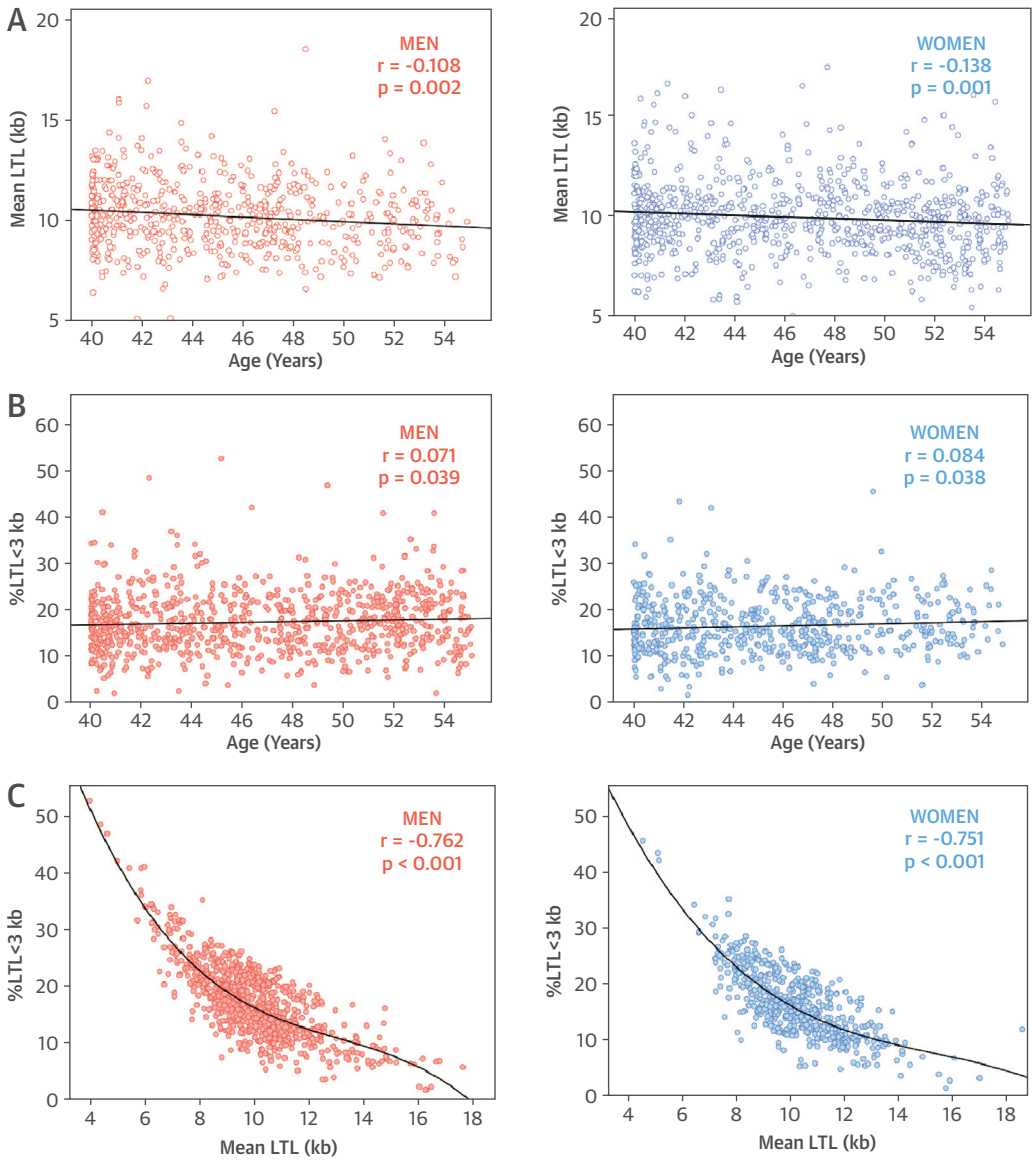
TELOMERE MEASUREMENTS IN LEUKOCYTES. Peripheral blood leukocytes were isolated with Histopaque (Sigma-Aldrich, St. Louis, Missouri), and cells were frozen in freshly prepared 90% FBS-10% DMSO and stored in liquid nitrogen until use. HT-QFISH in 96-well-plate format was performed on interphase nuclei of leukocytes to determine mean LTL and %LTL<3 kb (24). This technique uses high-throughput

TABLE 1 Plaque Presence and Total Plaque Burden

		Men (n = 848)	Women (n = 611)	Chi-Square p Value
CASC ≥1		203 (24)	25 (4)	<0.001
Carotid artery plaque		265 (31)	135 (22)	<0.001
Iliofemoral artery plaque		417 (49)	141 (23)	<0.001
Infrarenal aorta plaque		189 (23)	110 (18)	0.039
		Men (n = 416)	**Women (n = 158)**	**Student *t*-Test p Value**
Total plaque burden >0 (mm^3)	Tertile 1	16.2 ± 8.6	9.3 ± 4.2	<0.001
	Tertile 2	61.2 ± 19.3	26.9 ± 7.4	<0.001
	Tertile 3	263.5 ± 210.3	121.5 ± 100.4	<0.001

Values are n (%) or mean ± SD.
CASC = coronary artery calcium score.

FIGURE 1 Telomere Length in Circulating Leukocytes of Middle-Age Subjects Free From Established Cardiovascular Disease



(A) Scatterplot of mean LTL (kb) against age for men and women. **(B)** Scatterplot of %LTL<3 kb against age for men and women. **(C)** Scatterplot of %LTL<3 kb against mean LTL (kb) for men and women (cubic regression lines). N = 848 men; N = 611 women; %LTL<3 kb = percent short telomeres; LTL = leukocyte telomere length.

confocal microscopy to detect telomeres labeled with a fluorescent peptide nucleic acid probe against telomeric repeats. All samples were measured in duplicate, and mean LTL and %LTL<3 kb were calculated for each sample. Previous studies have used <3 kb as the cutoff for "short telomeres" in humans (18,25).

The intra-assay coefficient of variation was 6.3% for LTL and 10.5% for %LTL<3 kb. For 152 randomly chosen samples, duplicate measurements were repeated in a different plate to calculate the inter-assay coefficient of variation, which was 5.6% for LTL and 12.8% for %LTL<3 kb. The sample size (1,459) was sufficient to detect a standardized difference of 0.171 in LTL between groups with and without plaque with a statistical power of 0.9.

STATISTICAL ANALYSIS. All statistical analyses were performed using the Statistical Package for the Social Sciences (version 20.0, SPSS, IBM Corp., Armonk,

New York). The 3D ultrasound data on plaque burden were categorized into tertiles, and CACS was dichotomized into <1 and ≥1 Agatston units. Pearson correlation coefficients were calculated to assess the associations between telomere parameters and age. Linear regression lines were fitted to visually assess the associations between LTL and %LTL<3 kb with age and cubic regression lines for the associations between LTL and %LTL<3 kb. The chi-square test and Student *t* test were applied to assess differences in plaque presence and plaque burden between men and women. The association of other participant characteristics with mean LTL, %LTL<3 kb, or total plaque burden were assessed with analysis of variance and linear regression models. Independent associations of telomere parameters with plaque burden were assessed with linear regression models adjusted for other covariates.

Distinct circulating leukocyte subsets have different mean TLs (26–28). However, because analysis of our samples revealed no association between the percentage of different leukocyte subtypes and plaque burden, this variable was not taken into account as a confounder in the statistical analysis.

RESULTS

The baseline prevalence of subclinical atherosclerotic lesions in the 1,459 study participants is summarized in Table 1. Subclinical atherosclerosis was determined by vascular ultrasound as the presence of plaques in the infrarenal aorta, carotid arteries, or iliofemoral arteries (plaque presence and total plaque burden), or by CT determination of CACS. Plaques in all territories and CACS ≥1 were markedly more prevalent in men ($p < 0.001$). Moreover, total plaque burden in participants with observed plaques was much higher in men ($p < 0.001$). As expected, age in both sexes was inversely associated with LTL (men: Pearson's $r = -0.108$; $p = 0.002$; women: Pearson's $r = -0.138$; $p = 0.001$) (Figure 1A), and directly associated with %LTL<3 kb (men: Pearson's $r = 0.071$; $p = 0.039$; women: Pearson's $r = 0.084$; $p = 0.038$) (Figure 1B), and LTL was significantly longer in women than in men (women: 10.19 kb ± 1.69 vs. men: 9.96 kb ± 1.84; $p = 0.019$). We also found an inverse association between LTL and %LTL<3 kb in men (Pearson's $r = -0.762$; $p < 0.001$) and women (Pearson's $r = -0.751$; $p < 0.001$) (Figure 1C).

Table 2 shows the stratification of CVD risk factors in the study population by sex and plaque burden. A higher plaque burden was associated with a worse CVD risk-factor profile, especially among men. Unadjusted LTL differed significantly across the plaque burden categories in men, with the highest mean LTL value found in men with no plaques (p-trend = 0.033). LTL showed no significant association with plaque burden among women.

TABLE 2 Baseline Characteristics Stratified by Sex and Total Plaque Burden

Men	Plaque Burden (mm^3)*				Test for Trend p Value
	No Plaque (n = 391)	Tertile 1† (n = 137)	Tertile 2† (n = 138)	Tertile 3† (n = 141)	
Age, yrs	44.7 ± 0.2	46.0 ± 0.4	46.9 ± 0.4	49.1 ± 0.4	<0.001
LTL (kb)	10.17 ± 0.09	9.74 ± 0.17	9.71 ± 0.15	9.94 ± 0.15	0.033
%LTL<3 kb	16.9 ± 0.3	18.1 ± 0.6	17.4 ± 0.5	17.4 ± 0.6	0.38
BMI, kg/m^2	26.7 ± 0.2	26.9 ± 0.3	27.2 ± 0.3	27.7 ± 0.3	0.027
Systolic blood pressure, mm Hg	120 ± 0.5	121 ± 0.8	123 ± 1.0	124 ± 1.1	<0.001
Diastolic blood pressure, mm Hg	73 ± 0.4	75 ± 0.7	76 ± 0.8	78 ± 0.8	<0.001
Glucose, mg/dl	89.3 ± 0.5	90.7 ± 0.8	91.4 ± 0.8	95.8 ± 1.4	<0.001
Total cholesterol, mg/dl	196.8 ± 1.6	200.1 ± 2.9	203.8 ± 2.7	211.3 ± 2.9	<0.001
LDL cholesterol, mg/dl	129.0 ± 1.4	133.5 ± 2.6	137.7 ± 2.3	141.7 ± 2.6	<0.001
Triglycerides, mg/dl	97.1 ± 2.4	104.1 ± 4.3	106.6 ± 5.1	128.8 ± 6.6	<0.001
Ox-LDL, U/l	55.7 ± 0.9	60.7 ± 1.7	61.9 ± 1.6	64.6 ± 1.6	<0.001
Urinary isoprostanes, ng/ml	1.16 ± 0.03	1.04 ± 0.05	1.19 ± 0.08	1.28 ± 0.09	0.11
Waist circumference, cm	93.6 ± 0.5	94.7 ± 0.9	95.5 ± 0.8	97.6 ± 0.9	0.001
HbA_{1C}, %	5.3 ± 0.02	5.4 ± 0.03	5.4 ± 0.03	5.6 ± 0.03	<0.001
Alcohol consumption, g/day	11.7 ± 0.6	10.7 ± 0.9	12.9 ± 0.9	16.5 ± 1.4	0.001

Women	Plaque Burden (mm^3)*				Test for Trend p Value
	No Plaque (n = 440)	Tertile 1‡ (n = 52)	Tertile 2‡ (n = 52)	Tertile 3‡ (n = 54)	
Age, yrs	44.1 ± 0.2	45.3 ± 0.6	46.1 ± 0.6	48.1 ± 0.6	<0.001
LTL, kb	10.28 ± 0.08	10.09 ± 0.26	10.09 ± 0.21	10.38 ± 0.20	0.74
%LTL<3 kb	16.14 ± 0.28	16.72 ± 0.92	16.75 ± 0.73	15.60 ± 0.69	0.70
BMI, kg/m^2	23.78 ± 0.17	24.91 ± 0.51	23.83 ± 0.46	24.73 ± 0.60	0.09
Systolic blood pressure, mm Hg	108 ± 0.4	111 ± 1.5	112 ± 1.5	115 ± 1.5	<0.001
Diastolic blood pressure, mm Hg	68 ± 0.4	70 ± 1.3	71 ± 1.1	74 ± 1.2	<0.001
Glucose, mg/dl	84.9 ± 0.4	84.4 ± 1.0	85.1 ± 1.1	85.9 ± 1.3	0.79
Total cholesterol, mg/dl	192.9 ± 1.4	192.9 ± 4.5	198.0 ± 4.1	209.0 ± 4.2	0.004
LDL cholesterol, mg/dl	120.2 ± 1.3	124.6 ± 3.6	126.2 ± 3.5	137.0 ± 4.0	<0.001
Triglycerides, mg/dl	70.2 ± 1.3	74.2 ± 4.6	72.6 ± 4.1	74.0 ± 4.5	0.64
Ox-LDL, U/l	47.8 ± 0.7	52.5 ± 2.3	47.4 ± 2.0	53.5 ± 2.5	0.02
Urinary isoprostanes, ng/ml	0.89 ± 0.03	1.02 ± 0.09	0.78 ± 0.06	1.08 ± 0.14	0.07
Waist circumference, cm	79.5 ± 0.4	82.1 ± 1.3	81.8 ± 1.2	82.6 ± 1.5	0.037
HbA_{1C}, %	5.2 ± 0.01	5.2 ± 0.04	5.3 ± 0.04	5.4 ± 0.05	0.08
Alcohol consumption, g/day	4.9 ± 0.3	5.0 ± 0.8	4.6 ± 0.9	3.5 ± 0.7	0.53

*Plaque burden is calculated as a categorical variable (no plaque + sex specific tertiles). Results presented as mean ± standard error from analysis of variance test. †Tertile 1: 2.7 to 31.3 mm^3; tertile 2: 31.4 to 96.5 mm^3; tertile 3: 96.9 to 1,313 mm^3. ‡Tertile 1: 2.1 to 17.5 mm^3; tertile 2: 17.7 to 43.5 mm^3; tertile 3: 44.2 to 567 mm^3.

%LTL<3 kb = percent short telomeres; BMI = body mass index; HbA_{1C} = glycosylated hemoglobin; LDL = low-density lipoprotein; LTL = leukocyte telomere length; ox-LDL = oxidized low-density lipoprotein.

The %LTL<3 kb did not differ across plaque burden categories in men or women.

Sex- and age-adjusted associations between telomere parameters and CVD risk factors are shown in Table 3. The only direct significant associations after these adjustments were between %LTL<3 kb and urinary isoprostanes and serum ox-LDL in men (p = 0.007 and 0.008, respectively), and between %LTL<3 kb and serum ox-LDL in women (p = 0.001).

Associations between telomeres and atherosclerosis burden in the femoral and carotid arteries were investigated in 3 regression models: unadjusted, adjusted for age (and sex in nonstratified models), and also adjusted for 8 additional CVD risk factors (Table 4). The analysis considered plaque burden in these territories individually and in combination (total plaque burden). In the unadjusted model, LTL reached statistical significance as a determinant of femoral plaque and total plaque burden in men, and in the total sample. However, the association did not hold after adjustment for age and sex (Model 1) or additional CVD risk factors (Model 2). For %LTL<3 kb, none of the regression models showed significant associations for men, women, or the total sample.

TABLE 3 Association Between Telomere Measurements and CVD Risk Factors Stratified by Sex and Adjusted by Age*

	Dependent Variable: LTL			
	Men		Women	
Risk Factor	β Coefficient ± SE	p Value	β coefficient ± SE	p Value
BMI, kg/m^2	0.032 ± 0.019	0.09	0.021 ± 0.019	0.28
Waist circumference, cm	0.008 ± 0.006	0.23	0.004 ± 0.007	0.59
Overweight	0.170 ± 0.140	0.23	0.084 ± 0.151	0.58
Glucose, mg/dl	0.008 ± 0.005	0.15	0.012 ± 0.009	0.21
Hypertension	0.270 ± 0.175	0.12	0.529 ± 0.318	0.09
Current smoker	0.025 ± 0.162	0.88	0.153 ± 0.160	0.34
Yrs smoking	0.010 ± 0.007	0.14	0.002 ± 0.008	0.84
Urine isoprostanes, ng/dl	−0.043 ± 0.074	0.57	0.088 ± 0.098	0.37
Ox-LDL, U/l	−0.001 ± 0.003	0.99	−0.006 ± 0.004	0.20
Total cholesterol, mg/dl	0.003 ± 0.002	0.07	0.002 ± 0.002	0.43
Triglycerides, mg/dl	0.000 ± 0.001	0.65	0.000 ± 0.002	0.10
HbA_{1C}, %	0.191 ± 0.146	0.19	0.141 ± 0.219	0.52
Alcohol intake, g/day	−0.002 ± 0.005	0.66	−0.012 ± 0.011	0.26
	Dependent Variable: %LTL<3 kb			
	Men		Women	
Risk Factor	β Coefficient ± SE	p Value	β coefficient ± SE	p Value
BMI, kg/m^2	−0.030 ± 0.065	0.65	−0.076 ± 0.066	0.25
Waist circumference, cm	0.011 ± 0.023	0.63	−0.013 ± 0.025	0.60
Overweight	−0.040 ± 0.495	0.94	−0.087 ± 0.522	0.87
Glucose, mg/dl	−0.024 ± 0.019	0.22	−0.013 ± 0.032	0.69
Hypertension	−0.110 ± 0.618	0.86	−0.883 ± 1.099	0.42
Current smoker	−0.130 ± 0.573	0.82	0.053 ± 0.552	0.92
Yrs smoking	−0.031 ± 0.023	0.18	−0.016 ± 0.027	0.56
Urine isoprostanes, ng/dl	0.703 ± 0.261	0.007	0.276 ± 0.338	0.41
Ox-LDL, U/l	0.031 ± 0.012	0.008	0.055 ± 0.015	<0.001
Total cholesterol, mg/dl	−0.009 ± 0.007	0.16	0.007 ± 0.008	0.38
Triglycerides, mg/dl	0.000 ± 0.004	0.91	0.004 ± 0.008	0.61
HbA_{1C}, %	−0.939 ± 0.513	0.06	−0.765 ± 0.755	0.31
Alcohol intake, g/day	−0.005 ± 0.017	0.77	0.043 ± 0.038	0.25

*Data are regression β coefficients ± SE (standard error) in linear regression models.
CVD = cardiovascular disease; other abbreviations as in Table 2.

DISCUSSION

An association between short LTL and the occurrence of cardiovascular ischemic events has been described in several studies (13,14,29); however, information is still scarce on the relationship between LTL and subclinical atherosclerosis in subjects free from established CVD. The short telomere load correlates with the number of senescent cells (17), suggesting that an association between CVD and the number of short telomeres might be better than average LTL at predicting aging and associated atherosclerosis. However, previous studies have not investigated this possible association. Here, we analyzed LTL, as well as short telomere load (%LTL<3 kb), in a subset of 1,459 middle-aged CVD-free participants from the PESA cohort (2,21) (**Central Illustration**). Our analysis shows that neither high %LTL<3 kb nor short mean TL in circulating leukocytes is an independent determinant of subclinical atherosclerosis after adjusting for age or other well-known cardiovascular risk factors.

In 2 of 3 cross-sectional studies performed in relatively small cohorts (from 129 to 325 subjects), short LTL showed an association with asymptomatic atherosclerosis estimated by coronary artery calcification (30–32). In a 6-year prospective study of common carotid IMT in 768 participants, telomere shortening showed an association with increased subclinical carotid vascular damage and worse cardiovascular prognosis (33). In a cohort of 800 subjects, short LTL also emerged as a significant and independent predictor for the risk of advanced atherosclerosis and its complications (myocardial infarction and stroke), but was not associated with early atherogenesis (34). Similarly, the large-scale Asklepios study (2,509 relatively young volunteers free from established CVD) found no or only limited independent association between average LTL and IMT or plaque presence in carotid or femoral arteries in men and women (15). By analyzing different vascular territories in 1,459 PESA participants and performing a more extensive

characterization of subclinical atherosclerosis burden, which included not only the presence/absence of plaques, but also the continuous variable "plaque volume," our studies reinforce the notion that average LTL is not associated with subclinical atherosclerosis. In addition, we show for the first time that the abundance of short telomeres in leukocytes is not an independent determinant of subclinical atherosclerosis in carotid or femoral arteries. It would be important, however, to carry out longitudinal studies with the same participants analyzed herein to ascertain whether telomere attrition over time is associated with cardiovascular ischemic events. As expected, men in our cohort had a higher prevalence of subclinical atherosclerosis than women. This might explain the significant association of total plaque burden with LTL in men, but not in women, in the unadjusted model. Indeed, the overall young age of the study population might help to explain the lack of association between LTL and plaque burden, especially for women, who generally have a lower atherosclerosis burden than men of the same age.

Telomere dysfunction induces metabolic and mitochondrial compromise (35), and increased burden of oxidative stress can accelerate LTL attrition (12,29). Consistent with the results of multivariate regression analyses showing that femoral artery plaques are independently related to serum ox-LDL (36), we found significantly higher serum ox-LDL concentrations among participants with a higher plaque burden. Although we did not observe significant association between mean LTL and serum ox-LDL, interestingly, the %LTL<3 kb was significantly higher in men and women with higher serum ox-LDL levels. These results suggest that accumulation of short telomeres correlates with abnormal lipid accumulation in blood; therefore, longitudinal follow up of PESA participants will be of high interest.

TABLE 4 Association of LTL and %LTL<3 kb With Plaque Burden

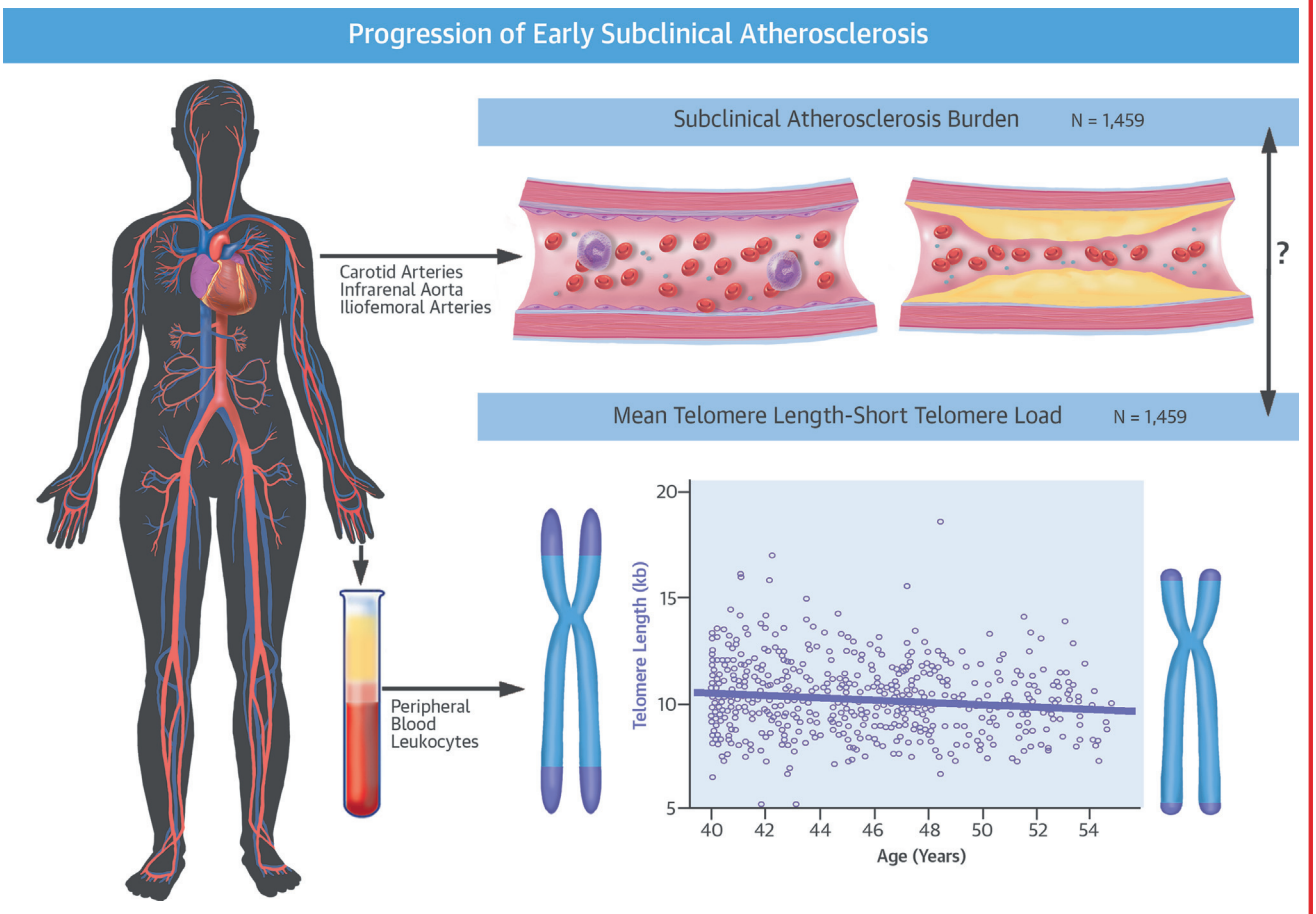
Total Sample (N = 1,349)	Linear Regression Model†	LTL (kb)		%LTL<3 kb	
		β Coefficient ± SE	p Value	β Coefficient ± SE	p Value
Total plaque burden*	Unadjusted	−0.121 ± 0.044	0.006	0.243 ± 0.152	0.11
	Model 1	−0.031 ± 0.048	0.52	−0.049 ± 0.167	0.77
	Model 2	−0.073 ± 0.050	0.15	0.008 ± 0.173	0.96
Carotid plaque burden*	Unadjusted	−0.021 ± 0.051	0.69	−0.071 ± 0.178	0.69
	Model 1	0.041 ± 0.052	0.44	−0.237 ± 0.181	0.19
	Model 2	0.016 ± 0.053	0.76	−0.206 ± 0.183	0.26
Femoral plaque burden*	Unadjusted	−0.108 ± 0-047	0.022	0.226 ± 0.163	0.17
	Model 1	−0.007 ± 0.052	0.89	−0.095 ± −0.179	0.59
	Model 2	−0.048 ± 0.054	0.37	−0.060 ± 0.185	0.75
Men (n = 780)	**Linear Regression Model†**	**LTL (kb)**		**%LTL<3 kb**	
		β Coefficient ± SE	p Value	β Coefficient ± SE	p Value
Total plaque burden*	Unadjusted	−0.116 ± 0.054	0.031	0.159 ± 0.189	0.40
	Model 1	−0.068 ± 0.057	0.23	0.012 ± 0.201	0.95
	Model 2	−0.106 ± 0.061	0.08	0.055 ± 0.211	0.79
Carotid plaque burden*	Unadjusted	−0.039 ± 0.064	0.54	−0.117 ± 0.227	0.61
	Model 1	0.001 ± 0.066	0.99	−0.220 ± 0.232	0.34
	Model 2	−0.023 ± 0.068	0.74	−0.190 ± 0.238	0.43
Femoral plaque burden*	Unadjusted	−0.111 ± 0.059	0.06	0.218 ± 0.207	0.29
	Model 1	−0.053 ± 0.063	0.40	0.053 ± 0.221	0.81
	Model 2	−0.092 ± 0.067	0.17	0.087 ± 0.233	0.71
Women (n = 569)	**Linear Regression Model†**	**LTL (kb)**		**%LTL<3 kb**	
		β Coefficient ± SE	p Value	β Coefficient ± SE	p Value
Total plaque burden*	Unadjusted	−0.030 ± 0.083	0.08	0.061 ± 0.282	0.83
	Model 1	0.055 ± 0.086	0.09	−0.114 ± 0.294	0.70
	Model 2	0.008 ± 0.090	0.09	−0.111 ± 0.308	0.72
Carotid plaque burden*	Unadjusted	0.016 ± 0.080	0.08	0.104 ± 0.272	0.70
	Model 1	0.073 ± 0.081	0.08	−0.002 ± 0.278	0.99
	Model 2	0.060 ± 0.083	0.08	−0.055 ± 0.284	0.85
Femoral plaque burden*	Unadjusted	0.004 ± 0.097	0.10	−0.182 ± 0.331	0.58
	Model 1	0.099 ± 0.100	0.10	−0.388 ± 0.343	0.26
	Model 2	0.032 ± 0.105	0.11	−0.336 ± 0.359	0.35

*Plaque burden is calculated as a categorical variable (0 value + sex-specific tertiles). †Linear regression models: Unadjusted = no adjustments; Model 1 = age (and sex in nonstratified models); Model 2 = model 1 + systolic blood pressure, diastolic blood pressure, hypertension diagnosis, fasting glucose, total cholesterol, smoking status, HbA_{1C}, ox-LDL, urine isoprostanes, and alcohol intake.

Abbreviations as in Table 2.

STUDY LIMITATIONS. First, our sample represents a homogeneous middle-aged and sociodemographic-specific population, and thus might not be representative of the general population; different results might be obtained in studies carried out with participants who are older (and thus have a larger extent of subclinical atherosclerosis) or who have different ethnic or demographic profiles (13,14,29). Second, our results are limited by the cross-sectional nature of the study. Prospective or longitudinal studies of large samples are clearly needed to establish with confidence whether there is an association between telomere parameters and the progression of subclinical atherosclerosis, and whether such an association could help identify subjects at high risk of developing CVD before symptoms appear (e.g., increased atherosclerosis progression, and risk of myocardial infarction and stroke in subjects with higher rates of LTL attrition and/or more accumulation of short telomeres over

CENTRAL ILLUSTRATION **Telomere Length and Subclinical Atherosclerosis: Design and Objectives**



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In a cross-sectional study, different vascular territories were analyzed by 2-dimensional and 3-dimensional ultrasound to quantify subclinical atherosclerosis burden and examine possible associations with mean telomere length and short telomere load in peripheral blood leukocytes examined by high-throughput quantitative fluorescence in situ hybridization.

time). Indeed, telomere shortening and accumulation of short telomeres over time has been proposed to be predictive of mortality in birds and mammals (37–39).

CONCLUSIONS

The results of our cross-sectional study of middle-aged men and women free from established CVD show that short mean telomere length and the amount of short telomeres (<3 kb) in leukocytes are not significant independent determinants of subclinical atherosclerosis burden. Future longitudinal studies are warranted to determine whether variation in telomere length over time is a predictor of subclinical atherosclerosis progression or the occurrence of cardiovascular ischemic events.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In a middle-aged population without clinical CVD, average LTL and short telomere load are not significant independent determinants of subclinical atherosclerosis.

TRANSLATIONAL OUTLOOK: Prospective studies are needed to determine whether subjects with higher rates of telomere length attrition or accumulation of short telomeres exhibit accelerated progression of atherosclerosis or an increased risk of ischemic events.

REFERENCES

1. Mozaffarian D, Benjamin EJ, Go AS, et al., American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2015 update: a report from the American Heart Association. Circulation 2015;131:e29–322.

2. Fernández-Friera L, Peñalvo JL, Fernández-Ortiz A, et al. Prevalence, vascular distribution, and multiterritorial extent of subclinical atherosclerosis in a middle-aged cohort: the PESA (Progression of Early Subclinical Atherosclerosis) study. Circulation 2015;131:2104–13.

3. Sanders JL, Newman AB. Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? Epidemiol Rev 2013;35:112–31.

4. Kovacic JC, Moreno P, Hachinski V, et al. Cellular senescence, vascular disease, and aging: Part 1 of a 2-part review. Circulation 2011;123:1650–60.

5. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer 2011;11:161–76.

6. Fyhrquist F, Saijonmaa O, Strandberg T. The roles of senescence and telomere shortening in cardiovascular disease. Nat Rev Cardiol 2013;10:274–83.

7. Broer L, Codd V, Nyholt D, et al. Meta-analysis of telomere length in 19,713 subjects reveals high heritability, stronger maternal inheritance and a paternal age effect. Eur J Hum Genet 2013;21:1163–8.

8. Blackburn EH, Epel ES. Telomeres and adversity: Too toxic to ignore. Nature 2012;490:169–71.

9. Codd V, Nelson C, Albrecht E, et al. Identification of seven loci affecting mean telomere length and their association with disease. Nat Genet 2013;45:422–7, 427e1–2.

10. Aviv A, Levy D. Telomeres, atherosclerosis, and the hemothelium: the longer view. Annu Rev Med 2012;63:293–301.

11. Aviv A. Genetics of leukocyte telomere length and its role in atherosclerosis. Mutat Res 2012;730:68–74.

12. Fuster JJ, Andrés V. Telomere biology and cardiovascular disease. Circ Res 2006;99:1167–80.

13. D'Mello MJ, Ross SA, Briel M, et al. Association between shortened leukocyte telomere length and cardiometabolic outcomes: systematic review and meta-analysis. Circ Cardiovasc Genet 2015;8:82–90.

14. Haycock PC, Heydon EE, Kaptoge S, et al. Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. BMJ 2014;349:g4227.

15. De Meyer T, Rietzschel ER, De Buyzere ML, et al., Asklepios Study Investigators. Systemic telomere length and preclinical atherosclerosis: the Asklepios Study. Eur Heart J 2009;30:3074–81.

16. Abdallah P, Luciano P, Runge KW, et al. A two-step model for senescence triggered by a single critically short telomere. Nat Cell Biol 2009;11:988–93.

17. Bendix L, Horn PB, Jensen UB, et al. The load of short telomeres, estimated by a new method, Universal STELA, correlates with number of senescent cells. Aging Cell 2010;9:383–97.

18. Vera E, Blasco MA. Beyond average: potential for measurement of short telomeres. Aging (Albany NY) 2012;4:379–92.

19. Baird DM, Rowson J, Wynford-Thomas D, et al. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat Genet 2003;33:203–7.

20. Hemann MT, Strong MA, Hao LY, et al. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 2001;107:67–77.

21. Fernández-Ortiz A, Jiménez-Borreguero LJ, Peñalvo JL, et al. The Progression and Early detection of Subclinical Atherosclerosis (PESA) study: rationale and design. Am Heart J 2013;166:990–8.

22. Sillesen H, Muntendam P, Adourian A, et al. Carotid plaque burden as a measure of subclinical atherosclerosis: comparison with other tests for subclinical arterial disease in the High Risk Plaque BioImage study. J Am Coll Cardiol Img 2012;5:681–9.

23. Stein JH, Korcarz CE, Hurst RT, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. J Am Soc Echocardiogr 2008;21:93–111; quiz 189–90.

24. Canela A, Vera E, Klatt P, et al. High-throughput telomere length quantification by FISH and its application to human population studies. Proc Natl Acad Sci U S A 2007;104:5300–5.

25. Elvsåshagen T, Vera E, Bøen E, et al. The load of short telomeres is increased and associated with lifetime number of depressive episodes in bipolar II disorder. J Affect Disord 2011;135:43–50.

26. Rufer N, Brümmendorf TH, Kolvraa S, et al. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. J Exp Med 1999;190:157–67.

27. Spyridopoulos I, Erben Y, Brummendorf T, et al. Telomere gap between granulocytes and lymphocytes is a determinant for hematopoetic progenitor cell impairment in patients with previous myocardial infarction. Arterioscler Thromb Vasc Biol 2008;28:968–74.

28. Spyridopoulos I, Hoffmann J, Aicher A, et al. Accelerated telomere shortening in leukocyte subpopulations of patients with coronary heart disease: role of cytomegalovirus seropositivity. Circulation 2009;120:1364–72.

29. Hunt SC, Kark JD, Aviv A. Association between shortened leukocyte telomere length and cardio-metabolic outcomes. Circ Cardiovasc Genet 2015;8:4–7.

30. Mainous AG III, Codd V, Diaz VA, et al. Leukocyte telomere length and coronary artery calcification. Atherosclerosis 2010;210:262–7.

31. Kroenke CH, Pletcher MJ, Lin J, et al. Telomerase, telomere length, and coronary artery calcium in black and white men in the CARDIA study. Atherosclerosis 2012;220:506–12.

32. Kark JD, Nassar H, Shaham D, et al. Leukocyte telomere length and coronary artery calcification in Palestinians. Atherosclerosis 2013;229:363–8.

33. Baragetti A, Palmen J, Garlaschelli K, et al. Telomere shortening over 6 years is associated with increased subclinical carotid vascular damage and worse cardiovascular prognosis in the general population. J Intern Med 2015;277:478–87.

34. Willeit P, Willeit J, Brandstätter A, et al. Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. Arterioscler Thromb Vasc Biol 2010;30:1649–56.

35. Sahin E, Colla S, Liesa M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. Nature 2011;470:359–65.

36. Langlois MR, Rietzschel ER, De Buyzere ML, et al., Asklepios Investigators. Femoral plaques confound the association of circulating oxidized low-density lipoprotein with carotid atherosclerosis in a general population aged 35 to 55 years: the Asklepios Study. Arterioscler Thromb Vasc Biol 2008;28:1563–8.

37. Heidinger BJ, Blount JD, Boner W, et al. Telomere length in early life predicts lifespan. Proc Natl Acad Sci U S A 2012;109:1743–8.

38. Vera E, Bernardes de Jesus B, Foronda M, et al. The rate of increase of short telomeres predicts longevity in mammals. Cell Rep 2012;2:732–7.

39. Fick LJ, Fick GH, Li Z, et al. Telomere length correlates with life span of dog breeds. Cell Rep 2012;2:1530–6.

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